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PYROLYSIS FOR ESTROGENS REMOVAL FROM WASTEWATER SOLIDS

by

Thomas Hoffman

A Thesis submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Master of Science and Engineering

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ABSTRACT
PYROLYSIS FOR ESTROGENS REMOVAL FROM WASTEWATER SOLIDS

Thomas Hoffman

Marquette University, 2015

Wastewater treatment processes are not designed to remove estrogenic micropollutants and therefore when wastewater biosolids are land applied estrogenic micropollutants are discharged to the environment. Release of estrogenic compounds has deleterious effects on the terrestrial environment. Public concern stemming from micropollutants may reduce the value of biosolids which are important to water resource reclamation facilities (WRRF) as by-products. The objective of this research was to evaluate pyrolysis, the partial decomposition of organic material in an oxygen-deprived system under high temperatures, as a sustainable solution to remove estrogenic compounds from biosolids while producing a useable soil conditioner called biochar. Batch pyrolysis was conducted at different temperatures (100-500°C) on anaerobically digested biosolids (ADB). Primary sludge (PS) and ADB samples were also collected from a WRRF to compare the estrogenicity of conventionally treated biosolids to pyrolyzed biosolids. These samples were extracted and analyzed via the yeast estrogen screen (YES) assay to quantify total estrogenicity. The YES assay utilizes a yeast strain, but the yeast is susceptible to toxicity from wastewater solids samples, causing estrogen response interference and test inaccuracy. Therefore, a cleanup method employing silica and alumina was developed and implemented prior to the YES assay to remove toxicity while maintaining estrogenicity. In the pyrolysis experiments, more than 95% of the estrogen equivalents were removed from biosolids at 400°C and higher temperatures. The biochar had significantly lower estrogenicity than PS and ADB. The lower estrogenicity in biochar could mean production of a more preferred product coming from WRRFs.

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Thomas Hoffman

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DEDICATION

I would like to dedicate this thesis to my family in Bethlehem, PA I wouldn't be able to move to a new city without your support and encouragement in everything I do and to Katie for all of your understanding and relief throughout this project.

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1 INTRODUCTION

1.1 Motivation

Biosolids can be a valuable byproduct from water resource recovery facilities (WRRFs) since the carbon and nutrient-rich solids are often land applied as a soil amendment. Public concern has risen regarding biosolids land application because of the presence of micropollutants (natural and anthropogenic organic compounds), including some estrogenic compounds and pathogens. Even though estrogenic compounds can be found at low concentrations, they can have negative environmental effects, which has encouraged interest in new biosolids management practices to mitigate concerns associated with biosolids land application.

Pyrolysis is an emerging biosolids management process that captures energy from organics to produce a combustible oil and gas, as well as biochar, a valuable solid product that can be used as a soil amendment and adsorbent. Reducing risks associated with biosolids by removing micropollutants and pathogens and preventing their release into the environment may be possible for WRRFs with production of biochar. No research has yet to be performed that quantifies the total estrogenicity of biosolids during pyrolysis. Total estrogenicity is important to quantify because it incorporates the estrogenic response of any remaining chemicals as well as transformation products that might be formed from parent compounds during pyrolysis.

1.2 Estrogenic Compounds in Biosolids

Natural estrogens as well as xenoestrogens are widespread in the environment and can have negative biological impacts once released into the environment. There are many pathways through which estrogenic compounds can enter the environment, and one of the

more important pathways is release from WRRFs. Because many estrogenic compounds are hydrophobic they can accumulate in the solids that are produced during wastewater treatment. Monitoring of these low concentration compounds can be difficult due to sludge matrix interference and toxicity, and measuring and adding together individual concentrations may not equal total biological response. Bioassays can be utilized to obtain a quantitative measure of biological activity. Other studies have utilized the yeast estrogen screen (YES) assay for measurement of biosolids total estrogenicity (Holbrook et al., 2002; Citulski & Farahbakhsh, 2012), but none have compared biosolids estrogenicity values to those of biochar.

1.3 Objectives

The goal of this research was to determine the impact of pyrolysis on estrogenicity of biosolids. Pyrolysis has been shown to remove some organic micropollutants (Bridle et al., 1990; Hu et al., 2007) and has potential to reduce total estrogenicity to yield a clean biochar product. The specific objectives of this research were to:

- Develop a simple method to analyze wastewater solids for estrogenicity via the YES assay
- Determine the effect of pyrolysis temperature on removal of estrogenic compounds from biosolids
- Determine the estrogenicity of wastewater solids, specifically primary sludge (PS) and anaerobically digested biosolids (ADB), compared to biochar

1.4 Approach

This research utilized the YES assay to quantify total estrogenicity in biosolids samples. Methods had to be developed to extract and clean the solids samples for the

YES assay. Because samples like PS and ADB were found to be inhibitory to the YES assay, the cleanup procedure had to be able to reduce toxicity while still recovering estrogenic compounds, as shown in the quality assurance/quality control (QA/QC).

The pyrolysis experiments were performed as batch experiments in a muffle furnace. The flasks used were studied under anoxic conditions at a variety of temperatures and times and analyzed for estrogenicity. Statistical software was then used to determine total estrogenicity and removal efficiency during pyrolysis.

Finally, PS and ADB samples were collected from a full-scale WRRF. These samples were extracted and analyzed for total estrogenicity and compared to the estrogenicity of biochar samples.

1.5 Thesis Structure

The following chapters provide an in-depth description of this research into estrogenicity of wastewater solids compared to biochar. A review of the previous relevant literature is provided in Chapter 2. Experimental methods from this project are shown in Chapter 3 and the results from those experiments are shown in Chapter 4. Finally, Chapter 5 offers conclusions to summarize the work and objectives tested. Standard operating procedures (SOPs) that were developed for the clean-up method and the YES assay are provided in the Appendix.

2 LITERATURE REVIEW

2.1 Biosolids

Biosolids refers to the organic solids produced during wastewater treatment. Worldwide at WRRFs, the solids created during treatment are sometimes stabilized through anaerobic digestion (AD) to produce biosolids (Carballa et al., 2007). Approximately 50% of the 7 million tons of biosolids produced at U.S. WRRFs are land-applied as a soil amendment (NEBRA, 2007). The benefit of land application of biosolids is that they contain carbon and other nutrients that improve soil quality and improve plant growth (US EPA, 2014). Biosolids may reduce chemical fertilizer requirements because of these properties and they also enrich the natural carbon cycle by placing organic carbon back into the environment (Lal, 2005). Biosolids can only be used as a soil amendment if they meet US Environmental Protection Agency requirements (40 CFR Part 503). The EPA designates biosolids as Class A and Class B for biosolids having non-detectable and detectable levels of pathogens, respectively (US EPA, 1994). Metals, pathogens, and nitrogen must be monitored in biosolids according to federal regulations (US EPA, 1994).

Biosolids are valued as a soil amendment for the reasons described above, but there is concern surrounding the land application of biosolids due to estrogenic compounds and other micropollutants associated with biosolids (McClellan & Halden, 2010; Holbrook et al., 2002; Kinney et al., 2008). AD, a common biosolids stabilization process, has not been shown to consistently remove many micropollutants, including pharmaceuticals and personal care products (PPCPs), and estrogenic compounds (Carballa et al., 2007; Citulski & Farahbakhsh, 2010; Hospido et al., 2010; Le-Minh et

al., 2010; Holbrook et al., 2002; McNamara et al., 2012). Public perception can be negative towards the idea of using biosolids as a soil amendment (Robinson et al., 2012), which is reflected by an organic grocery store, Whole Foods, completely banning produce from their store that was grown utilizing biosolids (NPR, 2014). Reducing the micropollutants could reduce the negative public opinion about biosolids use and thus increase the ability of WRRFs to convert this waste product into a value added product.

2.2 Estrogenic Micropollutants

Estrogenic compounds, including natural estrogens, such as estrone (E1), 17 β -estradiol (E2), and estriol (E3), and xenoestrogens, such as nonylphenol (NP) and 17 α -ethinyl estradiol (EE2), have raised concern due to their wide array of biological impacts and wide-spread occurrence in the environment. E2 is a steroidal hormone of the highest potency of the three natural estrogens and is the primary metabolite in a reproductive woman, while E3 is a metabolite of E1 and E2 (de Mes et al., 2005). E2 is synthesized naturally from both products of androstenedione, testosterone and E1, which is the predominant hormone in menopausal women.

Xenoestrogens are synthetic chemicals that bind to the estrogen receptor and modify endocrine pathways in the same manner as natural estrogens (Zacharewski, 1997). Estrogenic compounds, including synthetic compounds that were not designed to be estrogenic, can impact organisms because they diffuse into cells and bind with the estrogen receptor to form the hormone-receptor complex. The complex interacts with an estrogen response element of a target gene and increases gene expression for various proteins used in a diverse range of cellular processes. This process is shown in Figure 2.1. These processes include regulating the expression of certain genes and secretion of

specific hormones, and coordinating diverse processes (e.g., cell division, cell differentiation, and tissue organization) in organisms (Fang et al. 2000). The full estrogen mechanism is shown in Figure 2.1. Xenoestrogens are thought to act as estrogen receptor ligand mimics that bind to the receptor and modify endocrine pathways through the same process as a natural estrogen, but it is also possible that estrogen mimics elicit their effects independent of the estrogen receptor (Zacharewski 1997).

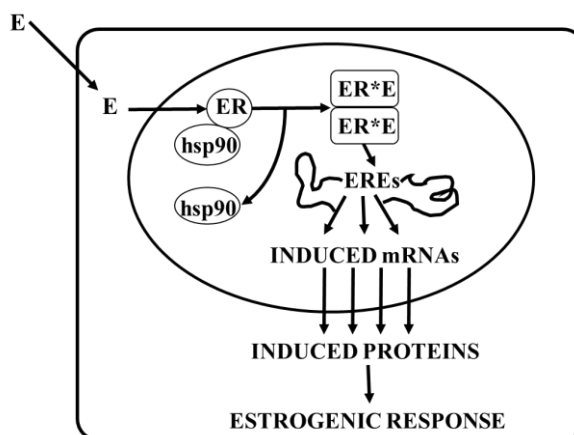


Figure 2.1: Estrogen receptor complex mechanism of action of estrogenic compounds. The estrogen (E) binds with the estrogen receptor (ER) causing the dissociation of protein 90 (hsp90). These complexes bind with each other and then bind to specific estrogen response element DNA sequences (EREs) which initiates gene transcription resulting in increased levels of gene expression (adapted from Zacharewski et al., 1997).

Chemicals such as bisphenol-A (BPA), polychlorinated biphenyls (PCBs), EE2 and NP are examples of xenoestrogens (Sonnenschein & Soto, 1998). EE2 is synthesized from E2 with an added ethinyl group that makes the compound more resistant to biodegradation compared to E2 and also has a higher estrogenic potency (Clouzot et al., 2008; de Mes et al., 2005). Xenoestrogens can come from residential and industrial sources and can be consumed and released through human waste and then reach WRRFs,

such as the compounds listed in Table 2.1, and are eventually released to the environment (Hamid & Eskicioglu 2012).

Table 2.1: Common estrogenic compounds and their sources (Kolpin et al., 2002)

Common estrogenic compounds	Sources
EE2 (ethinyl estradiol)	Contraceptive pill
nonylphenol	
nonylphenol monoethoxylate	Nonionic detergent
nonylphenol diethoxylate	metabolite (surfactant)
triclosan	Antimicrobial, disinfectant
(BPA) bisphenol-A	Plasticizer

The release of estrogenic compounds can occur through many avenues, whether from human or animal waste, or industrial waste streams. The many pathways can be seen below in Figure 2.2.

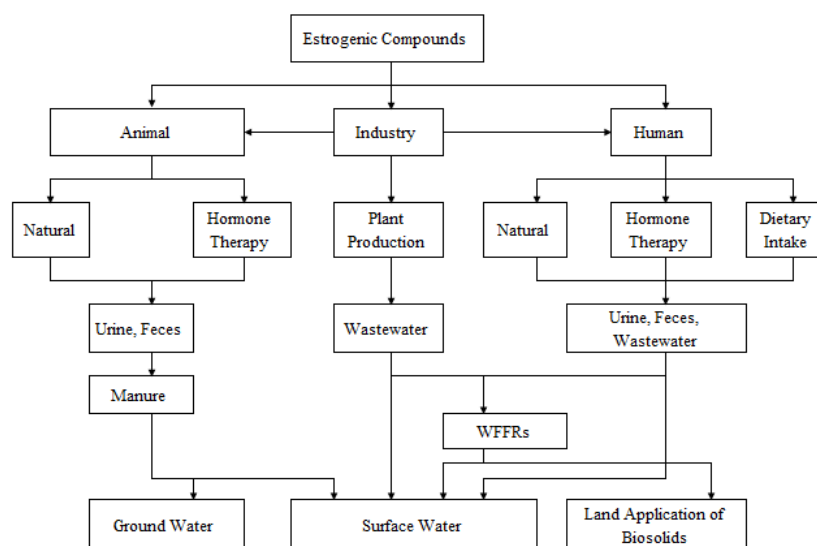


Figure 2.2: Pathways for release of estrogenic compounds (adapted from Hamid & Eskicioglu, 2012).

Human and animal excretion is thought to be the main source of steroidal hormones in aquatic environment (de Mes et al., 2005, Jobling et al., 2006). Estrogens are produced and released by both males and females with estimated excretion rates shown below in Table 2.2.

Table 2.2: Estimates of excretion of estrogens ($\mu\text{g/day/person}$) (Johnson et al., 2000)

	E1	E2	E3	Total
Males	1.6	3.9	1.5	7
Menstruating Females	3.5	8	4.8	16.3
Menopausal females	2.3	4	1	7.3
Pregnant Women	259	600	6000	6859

With this steady stream of estrogenic compounds to WRRFs, it's important to know where many of these compounds end up. Many estrogenic compounds are hydrophobic, with moderate to high log octanol-water partitioning ($\log K_{ow}$) values, and partition during wastewater treatment to biosolids that are sent for processes such as AD (Holbrook et al., 2002). Some estrogenic compounds and their K_{ow} values are shown below in Table 2.3.

Table 2.3: Estrogenic compounds with log K_{ow} (EPI Suite) and maximum concentrations found in biosolids

Compound	log K _{ow}	Biosolids (mg/kg)
Nonylphenol (NP)	4.48	483 ^a
Triclosan (TCS)	4.76	50 ^b
BPA	3.32	1.4 ^c
E2	4.01	20 (10 ⁻⁶) ^d
EE2	3.67	5 (10 ⁻⁶) ^d
E1	3.13	30 (10 ⁻⁶) ^d
^a Kinney et al., 2008; ^b Heidler & Halden, 2007; ^c Fromme et al., 2002; ^d Muller 2010		

McClellan & Halden estimated that over 200 metric tons of micropollutants are discharged due to biosolids land application every year in the U.S. (2009). These micropollutants include some estrogenic compounds which themselves have known deleterious impacts on the environment. Following land application of biosolids estrogenic compounds have been found to bioaccumulate in earthworms (Kinney et al., 2008). Looking at an aquatic environment, when approximately 5 ng/L of the synthetic estrogen 17- α -ethinyl estradiol (EE2) was added to a previously undisturbed lake, the fathead minnow (*Pimephales promelas*) population declined to near extinction (Kidd et al., 2007). Vajda et al. (2008) reported that the male population of white suckers (*Catostomus commersoni*) was only 20% of the total population downstream of a WRRF outfall that contained several estrogens. Upstream of the outfall the male population was 46% of the total population, a value typical of sustainable populations. A recent study has also shown that removal of estrogens from the incoming stream to an aquatic environment revived populations of species that had previously declined due to estrogenic activity (Blanchfield et al., 2015). These negative environmental impacts

highlight how important is it to monitor and mitigate impacts that environmental estrogens can have on the environment.

Measurement and monitoring of estrogenic compounds can be difficult because of their low concentrations and also because many solids samples are hard to analyze due to matrix interferences and toxicity. In general, when measuring individual compounds, liquid chromatography coupled with mass spectroscopy (LC-MS) is the most widely used technology (Hamid & Eskicioglu, 2012). For compounds such as estrogens, biological response, and not toxicity, is of main concern. Thus, measuring individual compounds may not always be the most efficient option for characterizing waste streams. When multiple estrogens and other chemicals are present at low levels, the total biological effect can be underestimated by adding together the effects of the individual compounds, which was shown in a study when eight estrogenic chemicals at significantly low concentrations produced a significantly larger biological effect when combined (Silva et al., 2002). Knowing the combined biological effect of samples to be discharged into the environment is crucial for understanding environmental impacts, and quantifying individual concentrations of chemicals within a sample will not yield this information.

Bioassays encompass any test that is used to determine the biological activity of a substance, usually using the live organism of concern in a natural environment (*in vivo*), or a microorganism surrogate in laboratory glassware with a similar target cell (*in vitro*). Cell-based *in vitro* bioassays have shown a high degree of sensitivity with results within a few days and reduces use of animals for *in vivo* testing. *In vitro* tests usually employ yeast as a surrogate cell for testing, while *in vivo* tests use small fish such as minnows.

Estrogenic *in vitro* bioassays fall into one of three categories: 1) estrogen receptor competitive binding assays that measure binding affinity of a chemical to the estrogen receptor; 2) cell proliferation assays that measure cell number increases of some target cell during the exponential phase and 3) reporter gene assays that measure estrogen receptor binding dependent transcriptional and translational activity (Fang et al., 2000). An example of a reporter gene assay, and what was used for this research, is the yeast estrogen screen (YES) assay. The YES assay developed by Routledge and Sumpter (1996) utilizes *Saccharomyces cerevisiae* transformed with the human estrogen receptor (hER) cDNA and an estrogen response element (ERE)-regulated Lac-Z reporter gene that encodes for β -galactosidase enzyme. This test has a lower limit of detection, 0.07 pM, than the other estrogen bioassays due to an increase in responsiveness (Zacharewski, 1997). The β -galactosidase is allowed to secrete into the media and metabolizes chlorophenol red- β -D-galactopyranoside (CPRG) changing the color from yellow to red (Routledge and Sumpter, 1996). The change in color is measured as an absorbance at 540 nm and dose-response curves are compared to an E2 standard dose-response curve. One major problem with the YES assay is that biosolids samples are often toxic to the yeast. A rapid clean-up method needs to be developed to be able to quantify estrogenicity of any biosolids sample (Citulski & Farahbakhsh, 2012). This bioassay allows a measurement of estrogenic activity of a sample as a surrogate for possible biological activity if released to a terrestrial environment.

2.3 Biosolids Management

Landfilling and incineration have been utilized as biosolids management practices as alternatives to land application but have been used less in the U.S. as more concern has

been placed on energy recovery (NEBRA, 2007). Landfilling interest is decreasing because it fails to recycle carbon and nutrients, falls short of producing a valued byproduct for WRRFs, and also simply moves micropollutants to another area (Campbell, 2000). Land application of biosolids has been shown to greatly reduce the carbon footprint when compared to landfilling (Peters & Rowley, 2009). It is unknown whether incineration removes estrogenic compounds although the compounds are most likely completely combusted given the right conditions, but incineration has some downfalls including negative public perception of air emissions and, sometimes, a negative energy balance (Campbell, 2000).

AD is most typically used because approximately 50% the organic fraction in biosolids can be converted to energy in the form of methane during typical operations. Although it may be more energetically favorable than incineration and landfilling, a consensus on the impact of AD on removal of total estrogenicity (combined estrogenic biological effect measured in E2 equivalents or EEq_s) has not been reached. Sludge matrix complexity and low micropollutant concentrations makes it difficult to quantify estrogens in biosolids, and, therefore, there are few studies that describe the impact of sludge stabilization on estrogen fate and varied results have been reported (Hamid & Eskicioglu, 2012). In batch AD experiments, more than 80% removal of natural estrogens was observed (Carballa et al., 2007). In full-scale digestion studies, no significant removal of E1, E2, and E3 were observed (Muller et al., 2010). Some studies even reported increased estrogenic response in full-scale mesophilic anaerobic digesters (Holbrook et al., 2002). AD potentially increased normalized EEq_s (moles of estrogens relative to solids content) because volatile solids were destroyed, but estrogenic

compounds were not; therefore, the estrogen mass relative to solids mass increased. Additionally, the estrogenic compound nonylphenol was found to be more hormonally active than its parent compounds, the nonylphenol ethoxylates, and was readily formed during AD; this could have also accounted for the increased estrogenicity observed after AD (Giger et al., 1984).

2.4 Pyrolysis

Pyrolysis is an abiotic thermal process that decomposes organic material at temperatures between 400 and 800°C in an oxygen-depleted environment (Laird et al., 2009). Pyrolysis can capture energy from the organic fraction in biosolids and also produce a valuable solid product. Biosolids pyrolysis yields a solid fraction (biochar), a gas fraction (py-gas), and a liquid fraction (py-oil), which are all usable byproducts (McNamara et al., 2014). The py-oil is formed when the heavier organics in the py-gas are allowed to condense. The py-gas and py-oil can each be combusted for energy (Menendez et al., 2002) with the organic fraction of the py-oil having a heating value comparable to conventional fuels like coal and the py-gas with possible heating values comparable to coke oven gas with optimization (Inguanzo et al., 2002).

Biochar is beneficial as a soil amendment because it can help improve soil drainage, plant growth, stress reduction, removal of bacteria from stormwater, and carbon sequestration (Barnes et al., 2014; Carey et al., 2013; Muhammad et al., 2014; Mohanty et al., 2014; Lehmann, 2007). Biochar has also been shown to be a capable sorbent of many different compounds including but not limited to: lead, atrazine, copper, zinc, chromium (VI), other heavy metals, naphthalene, BPA, EE2, phenanthrene, ammonia, and polycyclic aromatic hydrocarbons (PAHs) (Cao et al., 2009; Uchimiya et al., 2010;

Chen et al., 2011; Dong et al., 2011; Chen & Chen, 2009; Sun et al., 2011; Carey et al. 2013; Chen & Yuan, 2011).

Pyrolysis has been shown to remove organic pollutants from the solid phase by volatilization and decomposition reactions. A pilot-scale pyrolysis reactor operating for 30 min at 450°C removed 1.3 and 0.32 mg/kg of polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) respectively to below detection limits of less than 0.004 and 0012 mg/kg respectively (Bridle et al., 1990). Also, removal efficiencies of greater than 99.9% of dioxins and PCBs from sediments were seen in lab-scale pyrolysis at 800 °C with retention times of 30, 60, and 90 minutes (Hu et al., 2007). Pyrolysis can remove PCBs and dioxins from solid samples, and it is expected that pyrolysis can remove estrogenic compounds through a similar action because pyrolysis temperatures are typically higher than the melting temperatures of estrogenic compounds shown in Table 2.4.

Table 2.4: Common estrogenic compounds with boiling (T_B) and melting points (T_M) showing that all are below normal pyrolysis operating temperatures.

Common estrogenic compounds	T_M (°C)	T_B (°C)
NP (nonylphenol)	42*	295*
OP (4-octylphenol)	83	311
NP1EO (nonylphenol monoethoxylate)	116	370
Bisphenol-A	132	364
Triclosan [‡]	137	374
NP2EO (nonylphenol diethoxylate)	140	405
EE2 (ethinyl estradiol)	183*	411
E2 (17- β -estradiol)	222*	395
17- α -estradiol	222*	395
E1 (estrone)	260*	392
E3 (estriol)	290*	432
All data from <i>EPI Suite</i> estimations, except (*) from <i>EPI Suite</i> experimental database; [‡] estrogenic as shown by Stoker et al. (2010)		

2.5 Summary of Research Needs

Previous literature has suggested that organic micropollutants can be removed from solids during pyrolysis and that the process forms a valuable product in biochar. Pyrolysis that reduces the micropollutants, such as estrogenic compounds, found within biochar should significantly decrease public concern regarding land application of biosolids. In this study, experiments were conducted to determine if pyrolysis reduces the total estrogenicity of wastewater solids. The effect of temperature was studied and biochar samples were compared to other wastewater solids for estrogenicity. It was hypothesized that pyrolysis significantly removes estrogenic compounds, forming a biochar with a low total estrogenicity.

The following hypotheses were tested corresponding to the three research objectives:

Objective 1: Develop a simple method to analyze wastewater solids for estrogenicity via the YES assay.

Hypothesis: Silica and alumina can be used for cleanup columns to reduce yeast toxicity of sludge samples.

Objective 2: Determine the effect of pyrolysis temperature on removal of estrogenic compounds from biosolids.

Hypothesis: An increase in pyrolysis temperature will result in decreased biochar estrogenicity.

Objective 3: Determine the estrogenicity of wastewater solids, specifically PS and ADB compared to biochar.

Hypothesis: The pyrolysis product, biochar, will have a significantly lower total estrogenicity than PS and ADB.

3 METHODOLOGY

3.1 Wastewater Treatment Plant Sample Collection

Wastewater solids samples were collected at South Shore WRRF in Oak Creek, WI and analyzed for estrogenic equivalents (EEqs) to compare to biosolids samples that had been treated by pyrolysis. PS and ADB were collected with the assistance of the plant manager and immediately transported to the lab. Samples were placed in the freezer (-20°C) within one hour of collection and, once frozen, were subsequently lyophilized using a freeze dryer (Millrock BT Series, Kingston, NY). Freeze-drying the samples removed the water from the biosolids samples without having to use heat and risk estrogen loss through volatilization. Lyophilized samples were stored for approximately 24 hours at room temperature in acetone-rinsed aluminum tins prior to pyrolysis.

3.2 Pyrolysis of Wastewater Solids

3.2.1 Impact of Temperature on Estrogen Removal

Lyophilized ADB samples were homogenized using mortar and pestle and then a known mass (~300 mg) was added to sterilized Pyrex 150 mL Erlenmeyer flasks. The flasks were covered with aluminum foil and sparged with argon to remove headspace oxygen. The Water Quality Center had previously determined that sparging and covering the flasks with aluminum foil keeps the flask anoxic (Carey et al, 2013). Triplicate sparged flasks were placed in a muffle furnace at 100, 200, 300, 400, or 500 °C for 1 hr to determine the impact of temperature on estrogen removal. The flasks were then removed and allowed to cool in a desiccator. Final masses were recorded, solids were

transferred to acetone-rinsed aluminum tins, and solid samples were extracted as described in section 3.3.1.

3.2.2 Impact of Time on Estrogen Removal

Triplicate sparged flasks prepared as described in section 3.2.1 were placed in the muffle furnace for 5, 15, 30, and 60 minutes to determine the impact of pyrolysis reaction time on estrogen removal. Each time step was a separate experiment to eliminate the effect of a temperature drop when the muffle furnace had to be opened to remove samples. The flasks were allowed to cool in a dessicator until final masses were recorded and the solid sample was transferred to acetone-rinsed aluminum tins. The samples were then extracted following section 3.3.1.

3.3 Solid Sample Extraction and Processing

3.3.1 Hexane Extraction

Estrogens were extracted from lyophilized samples with hexane (HPLC Grade, >99%). A known mass of solid sample (0.1-0.5 g) was placed in aluminum-foil-capped 50 mL beakers, 25 mL of hexane was added to the beakers, and the beakers were ultrasonicated to strip chemicals off of the solids into the hexane (Branson 5800, Danbury, CT) for 30 min. The hexane extract was transferred to sterilized 100 mL glass bottles with screw-top caps. This extraction method was chosen because of its simplicity and use in previous studies (Holbrook et al., 2002; Citulski & Farahbakhsh, 2012).

3.3.2 Clean-up Columns

Some of the hexane extracts were toxic to yeast and, therefore, were processed through silica cleanup columns to remove toxicity prior to YES analysis. Cleanup columns were prepared by dry-packing 1 g of sodium sulfate, 1 g of 5%-activated silica

gel, 1 g of 5%-activated alumina, and 1 g of sodium sulfate into sterile 10 mL disposable syringes. To activate the columns for normal phase chromatography, 10 mL of methanol, followed by 10 mL of hexane were passed through the columns and wasted. Two mL of hexane extract was then added to the column followed by 10 mL of hexane rinse and elution by methanol (20 mL). The combined hexane rinse and methanol eluent was collected in sterilized, 50 mL beakers, evaporated to near-dryness and reconstituted in 2 mL of methanol that was pipetted into sterile amber glass vials and stored at 4°C until YES analysis. The cleanup columns and setup are pictured below in Figure 3.1. This cleanup method is modified for simplicity, safety, and time from one previously described by Citulski & Farahbakhsh (2012) who utilized SPE cartridges in conjunction with alumina and silica columns, and a 95:5 v/v hexane/toluene mix and ethanol for the solvents instead of hexane and methanol.



Figure 3.1: Cleanup columns setup can be seen (left) along with a closer picture of a silica and alumina column (right)

3.4 Yeast Estrogen Screen (YES) Assay

3.4.1 Procedure

The YES assay was performed on cleaned samples to quantify total EEq activity. Using the YES assay as opposed to measuring individual compounds accounts for a cumulative biological response of all hormonally active chemicals similar to what occurs in the environment (Holbrook et al. 2002). The procedure for the YES assay involves maintaining a pure culture of yeast (*S. cerevisiae*) that contains the human estrogen-receptor. A yeast culture is grown overnight which is then added to a new growth medium along with the yellow-colored chlorophenol-red-beta-galactopyranoside (CPRG) and pipetted into 96-well plates where the cleaned sample extract in methanol had previously been placed and evaporated to near dryness. In the presence of estrogenic compounds, the enzyme β -galactosidase is released to the surrounding environment and hydrolyzes CPRG, changing the well liquid color from yellow to red. The process is shown in Figure 3.2.

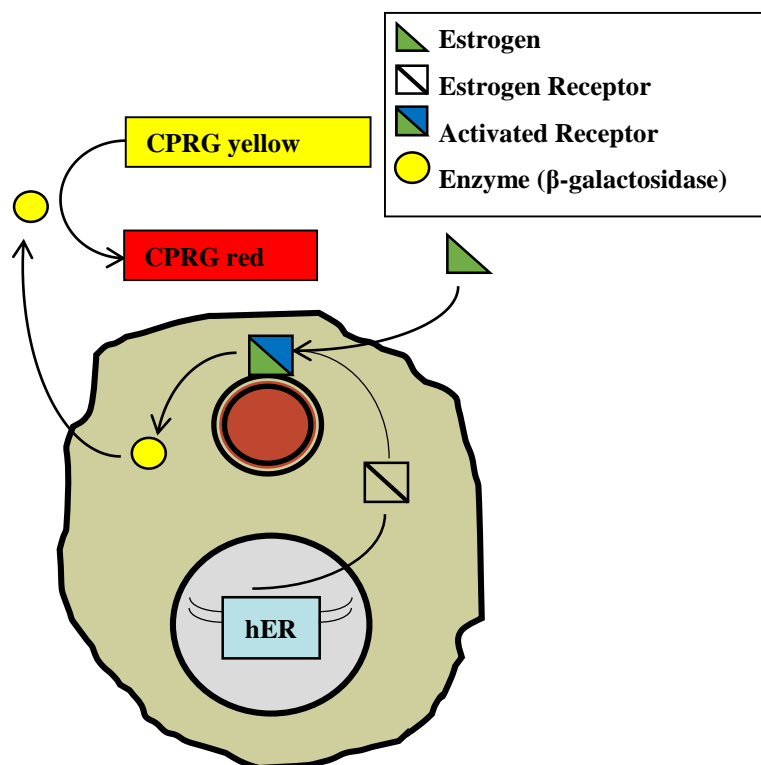


Figure 3.2: Mode of action of the yeast strain used for YES assay. The enzyme β -galactosidase is produced after an estrogen is bound to an estrogen receptor on a plasmid within the cell. The enzyme is released into the environment in the presence of estrogenic compounds and hydrolyzes the CPRG from yellow to red. Modified from Routledge & Sumpter (1996).

As the wells turn red measuring the absorbance values (at 540 nm) after 3-5 days allows for analysis of estrogenicity relative to a 17β -estradiol (E2) standard. The YES assay was performed according to the method of Routledge and Sumpter (1996) with a few modifications. (1) The absorbance at 620 nm was also measured to determine yeast growth over the incubation period, (2) 20 μ L from the dilution plate was added to the assay plate instead of 10 μ L, and (3) the stock E2 solution was prepared in methanol instead of ethanol. Absorbance was measured using a plate reader (SpectraMax, Molecular Devices, Sunnyvale, CA) and connected software (SoftMax Pro Data Acquisition and Analysis Software, Molecular Devices, Sunnyvale, CA).

3.4.2 Analyses

The absorbance at 540 nm and 620 nm was measured for initial and final values in order to analyze the extract samples for estrogenicity. Absorbance at 540 nm was transformed as shown in Eq. 1 to correct for background absorbance and turbidity as previously described by McNamara et al. (2012):

$$\text{Corrected } A_{540} = A_{540\text{total}} - A_{540\text{initial}} - 1.07 * [A_{620\text{total}} - A_{620\text{initial}}] \dots \dots \text{Eq. 1}$$

Where: corrected A_{540} is the absorbance used for dose-response analysis, $A_{540\text{total}}$ and $A_{620\text{total}}$ are the absorbance values after 3-5 days at 540 and 620 nm, respectively, $A_{540\text{initial}}$ and $A_{620\text{initial}}$ are the absorbance values initially after plate preparation.

Dose-response curves were generated from transformed absorbance values in GraphPad Prism 6.04 software. Using a nonlinear, variable slope, four parameter regression, the effective-concentration for 50% response (EC_{50}) was determined. EEqs were calculated as follows using Eq. 2:

$$\text{Solids EEqs} = \left(\frac{\text{E2 Standard } EC_{50}}{\text{Solid Sample } EC_{50}} \right) \dots \dots \dots \text{Eq. 2}$$

Where: solids EEqs are solids estrogenic equivalents (ng of E2-equivalents (E2-Eq) per gram of solids), E2 Standard EC_{50} and Solid Sample EC_{50} are the effective concentrations for a 50% response (ng E2/L and g solids/L, respectively).

3.5 Statistics

All samples were analyzed in triplicate to yield averages and standard deviations. GraphPad Prism 6.0 was used for all statistical analysis including t-tests to compare two data sets and analysis of variance (ANOVA) to compare more than two data sets. All statistics reported for significant differences are analyzed at a 95% confidence interval (p-value < 0.05).

3.6 Quality Assurance/Quality Control

3.6.1 Variance of Processing Stages

Experiments were performed to determine the reproducibility of the YES assay cleanup procedure, and specifically determine the variability in the i) extraction step, ii) clean-up column step, and iii) plating step. A PS sample was hexane-extracted in triplicate and subsequently cleaned and analyzed with the YES assay to determine reproducibility of the entire process. One hexane-extracted PS sample was cleaned with three separate columns and then analyzed via the YES assay to determine reproducibility of cleanup columns. One cleaned extract was plated in the 96-well plate in triplicate to determine reproducibility of plating the assay.

3.6.2 Estrogenic Equivalents Recovery

Triplicate spike and recovery experiments were performed with a known mass of the YES assay E2 standard added to a clean-up column to determine if estrogens spiked onto columns were recovered. The column procedure described above was followed, except instead of adding the 2 mL of solids extract during the cleanup process, 2 mL of the E2 standard in hexane was added and eventually reconstituted in 2 mL of methanol.

4 RESULTS & DISCUSSION

4.1 Method Development and Quality Assurance/Quality Control

4.1.1 Clean-up Columns

Clean-up methods are often required for sludge samples to reduce interference and toxicity to yeast in the YES assay (Citulski & Farahbakhsh 2012). A modification of the cleanup method of Citulski & Farahbakhsh (2012) was successfully used for rapid throughput. PS samples that were not put through cleanup columns yielded no estrogenic response because of yeast growth inhibition and receptor blocking, whereas cleaned PS samples produced a significant estrogenic response, as shown in Figure 4.1. PS, which has not undergone biological treatment, is often the most toxic of WRRF biosolids samples (McNamara 2012). ADB samples showed a different inhibitory effect to the YES assay. Instead of inhibiting yeast growth, the ADB samples most likely inhibited some estrogens from binding to the estrogen receptor except at higher concentrations, as evident in Figure 4.1.

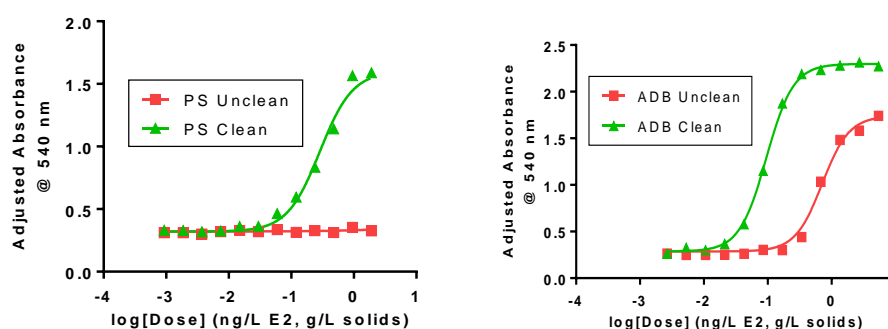


Figure 4.1: Cleanup columns reduce toxicity to the yeast. The PS sample (left) inhibited yeast at all tested concentrations and only yielded a response after the cleanup column. The ADB sample (right) was not as toxic as PS but clearly shows an inhibited curve when compared to the full s-curve of the cleaned ADB sample.

Cleanup columns utilized 5% activated alumina and silica because, as shown in Figure 4.2, the unactivated column did not retain the E2 standard while the activated column showed a measurable E2 response. Overall, the cleanup method was a rapid means to successfully reduce sludge extract toxicity towards yeast in the YES assay.

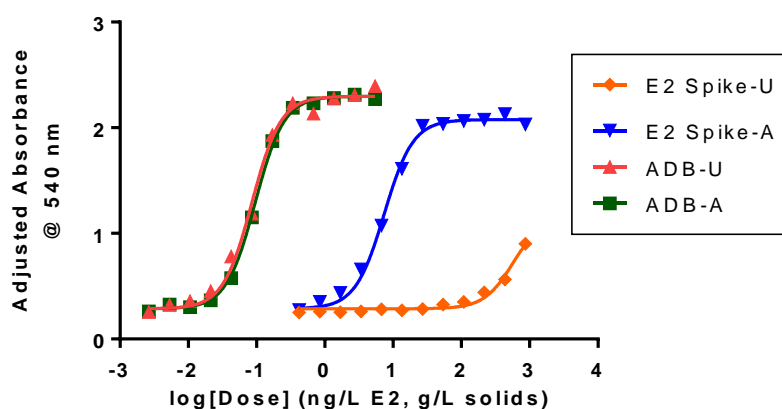


Figure 4.2: Using activated silica and alumina in cleanup columns (A) improved the recovery of E2 through the column compared to unactivated columns (U).

4.1.2 Method Reproducibility

Samples were extracted, cleaned-up, and plated in triplicate to determine the variability through each processing stage. The average \pm standard deviation EE_q values for PS plated in triplicate are shown in Figure 2 in units of ng E2-E_q/g solids. The values presented for each of the three stages were analyzed via one-way ANOVA and were not significantly different (p -value = 0.437), suggesting that the method developed is reproducible and no particular step substantially increases variability. As seen in Figure 3, the standard deviation of EE_qs for the extraction step was the highest, with a coefficient of variation (COV) of 21%, while the clean-up columns and plating steps have a tighter standard deviation with COVs of 7% and 4%, respectively. The extraction step

should have the highest variability because it incorporates the other two steps while the plating step merely has one step encompassing its variation.

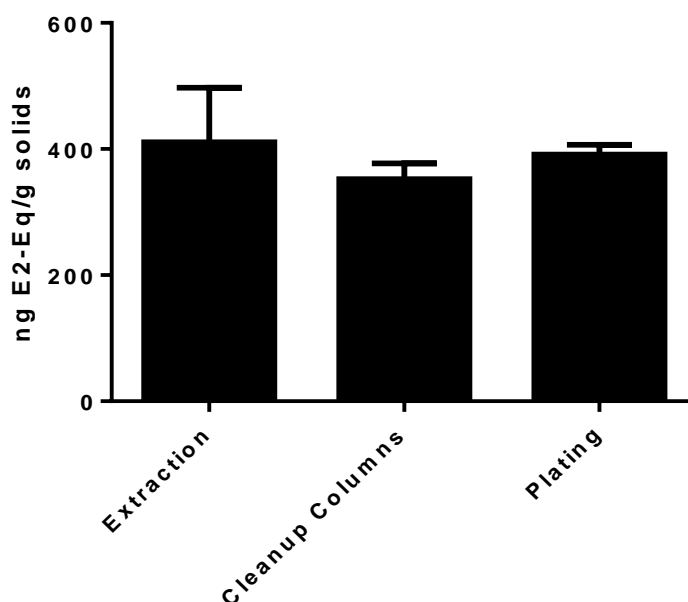


Figure 4.3: Reproducibility of YES assay processing stages. The extraction step showed the greatest COV (21%), while cleanup columns (7%) and plating (4%) showed less variance across samples. Bars represent the average of triplicate samples and error bars represent the standard deviation.

4.1.3 17 β -Estradiol Recovery

Spike and recovery tests with E2 were performed on the cleanup columns to determine if EEqs were lost in the column. The spiked E2 in triplicate was significantly different from the E2 standard (p-value = 0.028, t-test) with the column sample having an average of 31% of the standard's EEqs as shown in Figure 4.4. Although the spiked E2 was different from the standard, this test shows that an estrogenic response is recovered

and this method can be used to compare samples that were all processed in the same manner but may not produce accurate absolute quantifications.

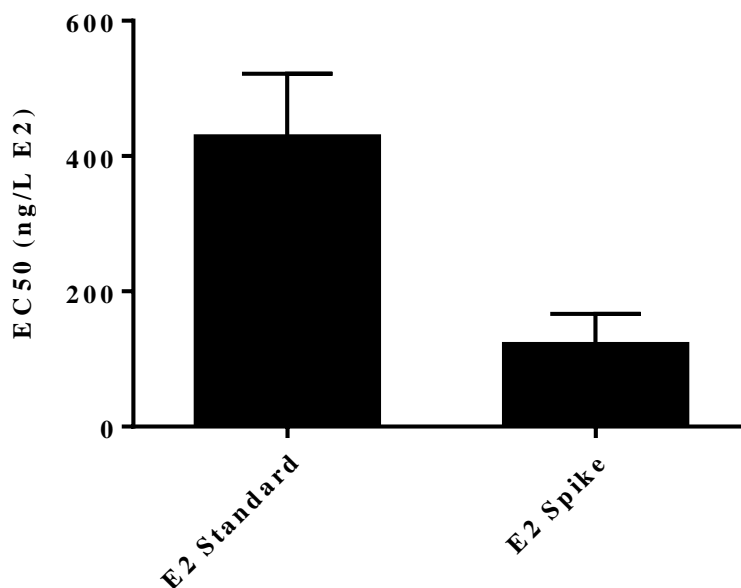


Figure 4.4: E2 recovery through the cleanup columns. The E2 standard column shows the EC-50 value of the standard solution used for the YES assay, while the E2 spike column shows the standard after being placed through the cleanup columns.

4.2 Impact of Pyrolysis Temperature on Estrogen Removal

Pyrolysis temperature has a large impact on the removal of EEEs from biosolids samples. EEE removal increased as pyrolysis temperature increased, with almost complete removal (>95%) at or above 400 °C (see Figure 4.5). The effectiveness of pyrolysis on EEE removal is not surprising given that the melting points of several common estrogenic compounds (presented in Table 2.4) are below 300°C. The higher temperatures allow for all of the compounds to make the necessary transition to their

liquid phase and eventually volatilize from the biochar. This volatilization step then allows the compounds to transition to the gas phase.

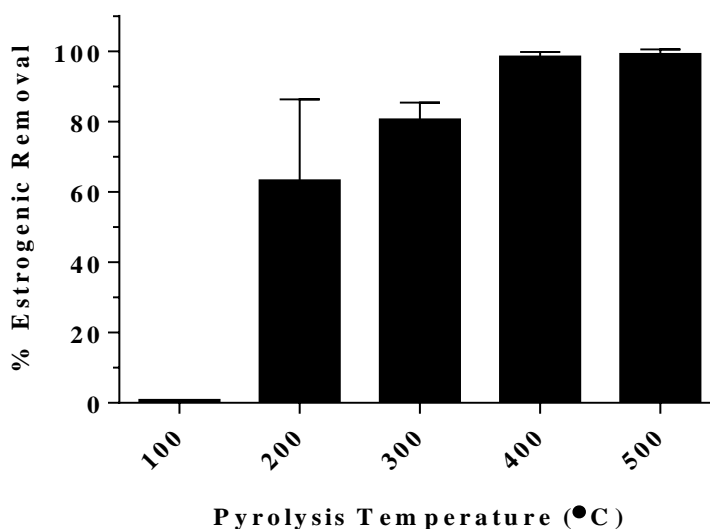


Figure 4.5: Removal of estrogenicity increased as pyrolysis temperature increased with greater than 95% removal above 400°C. Bars represent the average removal from triplicate samples and error bars represent standard deviation.

After initial volatilization from the biochar, the estrogenic compounds could either partition to the py-oil or py-gas, or even be partially or completely transformed through thermal decomposition. More research is needed to determine if transformation is occurring. Because the YES assay measures estrogenic response, and not individual estrogenic compounds, it takes into account any transformation products that may also be estrogenic and residing in the final biochar product. It can be concluded that, at 400°C, any parent estrogenic compounds and any residual estrogenic metabolites are removed from biochar. The estrogenic compounds and any transformation products that may arise and transfer into the py-gas or py-oil would most likely be oxidized when these high

energy byproducts are subsequently combusted in boilers or internal combustion engines. Commonly studied pyrolysis temperatures are above 400°C and sometimes are significantly higher than the temperatures used in this study (Laird et al. 2009), suggesting that the pyrolysis process, if used in full-scale, would remove greater than 95% of the estrogenic load in biosolids.

4.3 Impact of Pyrolysis Time on Estrogen Removal

Pyrolysis residence time was shown to be an important factor in estrogenic removal. No significant removal was shown until a 60 minute residence time (p-value = 0.0002). Although statistically no significant removal was shown, it can be seen in Figure 4.6 that there appears to be an increase in removal given more time in the reactor. At 5 minutes there was an apparent increase in estrogenicity in one sample which yielded a large standard deviation, but still the sample was not significantly different from the influent (p-value = 0.5375).

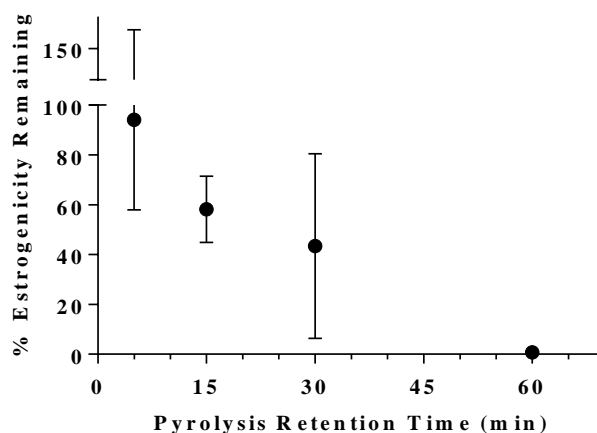


Figure 4.6: Estrogenicity remaining from 5 to 60 minute retention times. Results are shown as averages of triplicate data with error bars as standard deviation. No significant removal is seen until a 60 minute retention time.

4.4 Comparison of Biochar to Other Wastewater Solids

PS and ADB are estrogenic, see Figure 4.6, and thus additional treatment is required to remove estrogenic compounds from these biosolids samples. Pyrolysis of ADB substantially reduced EEq in the resulting biochar product (see Figure 4.7). The PS and ADB samples taken from a WRRF, as well as laboratory produced biochar (B) made from pyrolysis of ADB, were all analyzed for total EEq. PS and ABD EEq values of 491 and 438 ng E2-Eq/g solids, respectively, are near values found in literature for other biosolids samples and complement the inconsistent removal efficiencies found through AD (Hamid & Eskicioglu, 2012). The PS and ADB triplicate samples were not significantly different from each other (p-value = 0.6788, t-test). Biochar, however, had significantly lower EEq (p-value = 0.0005, ANOVA) as each 500°C sample was below 12 ng E2-Eq/g solids. Assuming 50% of the 7 million tons of biosolids produced each year in the U.S. are land-applied and have average EEq values for ADB found in this study (438 ng E2-Eq-g solids), performing pyrolysis at 500°C (to 12 ng E2-E2/g solids) would reduce the estrogenic loading from approximately 1,400 kg E2-Eq to 7 kg E2-Eq when land applying biochar.

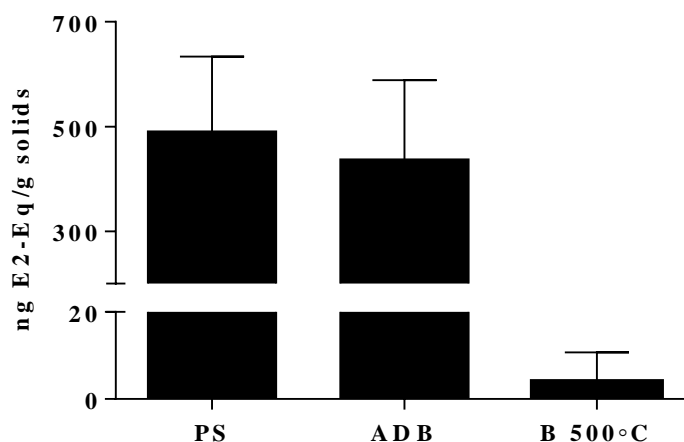


Figure 4.7: Comparison of total estrogenicity of biochar (B 500°C) to primary sludge (PS) and anaerobically digested biosolids (ADB).

4.5 Environmental Implications

Overall, this research has shown that pyrolysis is a treatment option that removes the majority of EEqs from biosolids, with greater than 95% removal above 400°C. This could reduce the spread of estrogenic compounds to the environment. The questions of where the estrogenic compounds go and if they are transformed remain to be answered and are important steps for a complete understanding of how pyrolysis can contribute to WRRF biosolids management. If estrogenic compounds are not destroyed then they will be transferred into the py-gas or py-oil destined for combustion. If complete combustion occurs, then the estrogenic compounds in the py-oil or py-gas would be mineralized. The biochar product is a less-hormonally active product than biosolids and thus less likely to have negative public opinion related to land application. Future work should further investigate the effect of reaction time on estrogenicity removal as well as a mass balance on estrogenicity of AD and heat drying to compare to pyrolysis. These results would inform decisions related to the best processes available to WRRFs. Overall, this research

shows that land application of biochar compared to biosolids would reduce the release of estrogenic compounds into the environment.

5 CONCLUSIONS

The goal of this research was to determine the impact of pyrolysis on estrogenic compounds in wastewater solids. The YES assay coupled with a simple extraction and cleanup process was used to determine the estrogenicity of PS, ADB, and biochar at different temperatures. The following conclusions are based on the reported laboratory-scale experiments performed at the Marquette University Water Quality Center.

- 1 The cleanup method developed for this research reduces wastewater solids toxicity and is a reproducible and simple processing step that allows for the analysis of estrogenicity of solids samples via the YES assay.
- 2 Pyrolysis of biosolids can reduce the release of estrogenic compounds to the environment. Performing pyrolysis of ADB at 500°C would reduce the estrogenic loading by approximately 99% when land applying biochar.
- 3 Pyrolysis temperature greatly impacts the removal of estrogenic compounds from biosolids. Removal of estrogenicity to greater than 95% was achieved above 400°C.
- 4 Biochar samples had significantly lower total estrogenicity than PS and ADB, while PS and ADB had estrogenicity values that were not significantly different from each other.

In summary, pyrolysis can reduce the spread of estrogenic compounds into the environment and is a viable biosolids management technology alternative to land application of biosolids not treated by pyrolysis.

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APPENDIX

A: Standard Operating Procedure (SOP) for Yeast Estrogen Screen (YES) Assay

Biosolids Preparation

Department of Civil, Construction, & Environmental Engineering

Marquette University, Water Quality Center

Prepared June 2014

Preparation Considerations

- Glass materials should be used whenever possible as plastics may interfere with the YES assay.

- Become familiar with the hazards associated with solvent, CPRG, and E2 handling.

¹ Sterilized by Autoclave and also 1 hour in 550 °C muffle furnace, covered in aluminum foil

² Sterilized by Autoclave, covered in aluminum foil

³ Rinsed with acetone and allowed to dry

1.0 SCHEDULE

Day 1: (4 + 24 hr wait)

Freeze-drying

Extraction materials sterilization

Streak new yeast plate (or prior)

Solution prep (or prior)

Day 4: (1-2 hr)

Assay medium culture

Dilution plate

Assay plate

Add assay medium

Day 0 readings

Day 2: (3 + 0.5 hr wait)

Extraction start

Clean-up materials sterilization

Sterilize beakers for cultures

Extraction finish

Day 7-9: (30 min)

Day 3-5 readings

Analysis

Day 3: (3 + 8 hr wait)

Columns

Clean-up start

24-hr culture

Prep assay materials

Clean-up finish

2.0 SAMPLE EXTRACTIONS

Materials and Equipment:

- Sterile¹ 50 mL beakers (1 for each sample)
- Sterile¹ glass bottles with screw-top caps² (1 for each sample)
- Sterile¹ freeze-drier bottles
- Mortar and pestle³
- Lab grade hexane (25 mL for each sample)
- Freeze-drier
- Sonicator

Freeze Drying:

1. If sample to be analyzed contains a large amount of water, such as primary or anaerobically digested sludge, it must be freeze-dried. Place a well-mixed aliquot of the sample into the sterile freeze-drier bottles (about 10-25 mL into each depending on how much is needed, but the more sample volume in each the longer it will take). Place aluminum foil over the top of the bottle and then place into the freezer for about 6-12 hours.
2. Once frozen place the tops onto the bottles with the glass attachment as well as the paper filter and red O-ring.
3. Turn the freeze-drier on with all valves closed and no water inside the cooling chamber. Attach the bottles to the freeze-drier and make sure the pressure goes below 500 mT. If the pressure does not drop low enough make sure all the bottles and hoses are attached tightly.
4. Leave freeze-drier run for about 24 hours (~ 36 mL per each bottle)
5. Once finished there should be no ice left in the solid sample. Place the remaining solids into tins that are labelled appropriately.

Extraction:

1. Record mass of sterile beaker without aluminum foil cover
2. Record mass of solid sample plus beaker, label with permanent marker on tape on aluminum foil

- a. 0.1 g of primary sludge or anaerobically digested solids (homogenized with mortar and pestle)
 - b. 0.5-1 g of biochar or Milorganite
3. Pipette 25 mL of hexane into beaker, cover with aluminum foil with appropriate label
4. Place immediately into sonicator and run for 30 minutes
5. After sonication, remove aluminum foil and record mass of hexane extract plus beaker
6. Immediately pour extract into glass bottle, trying to leave the solid residual in the beaker, and screw on cap. Label the bottle appropriately with tape and store at 4 °C.

3.0 EXTRACT CLEAN-UP

Materials and Equipment:

- Sterile¹ beakers (1 for each extract)
- 250 mL beakers
- Sterile 10 mL syringes
- Sterile¹ amber glass vials and screw-top caps² (1 for each extract)
- Ring stand, iron ring, test tube rack, 25 mL burette, burette clamps
- Methanol (~32 mL per extract)
- Hexane (~20 mL per extract)
- Sodium sulfate (Na₂SO₄) (2 g per extract)
- Activated silica, 5% water by wt. (1 g per extract)
- Activated alumina, 5% water by wt. (1 g per extract)
- Glass wool

Columns:

- Layer sodium sulfate, activated silica, and activated alumina into 10 mL syringe.

Top: 1 g sodium sulfate

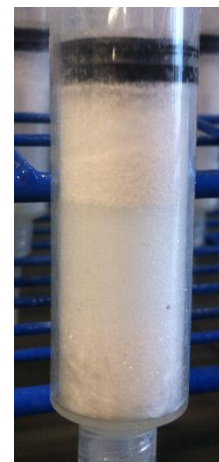
1 g activated silica*

1 g activated alumina*

1 g sodium sulfate

Bottom: Rolled up glass wool, enough to cover the bottom

*interchangeable

Clean-Up:

- Set-up the clean-up columns similar to what is shown below utilizing the test tube rack, ring stand, iron ring, and 25 mL burette and burette clamp.



- Drop solvents through the column in the following order without letting the column dry, collecting the eluent in 250 mL beakers
 1. 10 mL methanol
 2. 10 mL hexane
 3. 2 mL of extract
 4. 10 mL hexane
 5. 20 mL methanol

- Begin collecting the eluent in labeled sterile beakers at step 4 after adding the 2 mL of extract.
- Once all of the eluent is collected place the beakers into a hood and allow all liquid to evaporate (~6-8 hours)
- Reconstitute by glass pipetting ~1 mL methanol into the beakers, swirl around and then glass pipette the cleaned extract into sterile amber glass vials
- Repeat about 2 more times and measure total mass added into vial, label appropriately with tape, then store at 4 °C.

...FOLLOW YES ASSAY SOP

B. Standard Operating Procedure (SOP) for Yeast Estrogen Screen (YES) Assay

Department of Civil, Construction, & Environmental Engineering

Marquette University, Water Quality Center

Prepared February 2015

Preparation Considerations

- Glass materials should be used whenever possible as plastics may interfere with the YES assay.

- Become familiar with the hazards associated with solvent, **CPRG**, and E2 handling.

-All samples involving yeast cultures must be autoclaved before any disposal

-Refer to lab safety procedures for all disposal methods

¹ Sterilized by 1 hour in 550 °C muffle furnace, covered in aluminum foil

² Sterilized by Autoclave, covered in aluminum foil

³ Rinsed with acetone and allowed to dry

⁴ Rinsed with methanol and allowed to dry

Summary of Method – This bioassay uses recombinant yeast integrated with the DNA sequence of the hER and expression plasmids. The expression plasmids carry the reporter gene *lac-Z* and encode the enzyme β -galactosidase. When a chemical binds to the hER, gene transcription initiates the expression of the reporter gene *lac-Z*, secreting β -galactosidase into the medium which metabolizes the chromogenic substrate chlorophenol red- β -D-galactopyranoside (CPRG). Metabolized CPRG forms a red product and the absorbance of this product is measured using an optical density microplate reader. For more detailed information see:

Routledge, E.J., Sumpter, J.P., 1996. “Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen.” *Environmental Toxicology and Chemistry* 15 (3), 241-248.

1.0 SCHEDULE

Day 1:(4 + 24 hr wait) wait)

Freeze-drying

Extraction materials sterilization

Streak new yeast plate (or prior)

Solution prep (or prior)

Day 2: (3 + 0.5 hr wait)

Extraction start

Clean-up materials sterilization

Sterilize beakers for cultures

Extraction finish

Day 3: (3 + 8 hr

Columns

Clean-up start

24-hr culture

Prep assay materials

Clean-up finish

Day 4: (1-2 hr)

Assay medium culture

Dilution plate

Assay plate

Add assay medium

Day 0 readings

Day 7-9: (30 min)

Day 3-5 readings

Analysis

2.0 YEAST STORAGE AND PREPARATIONS

Materials and Equipment:

- Sterile¹ 250 mL flask
- Sterile petri dishes
- Water bath at 50°C
- Cryovials

2.1 Minimal Medium Agar Plate Preparation - makes four agar plates for short-term yeast storage

1. Place the following ingredients into a sterile flask:
 - 1 g bacteriological grade agar
 - 90 mL minimal medium
2. Autoclave at 121°C for 10 minutes
3. Cool to 50°C
4. Place glass container into 50°C water bath to prevent agar from setting
5. Add the following growth medium components to glass container while in water bath:
 - 10 mL glucose
 - 2.5 mL L-aspartic acid
 - 1 mL vitamin solution
 - 0.8 mL (800 µL) L-threonine solution
 - 250 µL copper (II) sulfate
6. Swirl to mix
7. Flame lip of container
8. Pour ~20 mL into first plate (do not remove plate lid until ready to pour contents)
9. Flame lip of container
10. Pour second plate
11. Repeat flaming/pouring technique for rest of plates
12. Allow plate to harden
13. Turn plate upside down to prevent condensation from dripping onto agar
14. Store plates in refrigerator

2.2 Short-term Yeast Storage Plate Preparation - Streak a new yeast storage plate every 30 days to ensure health of short-term yeast stock

1. Remove a new, unstreaked agar plate from refrigerator
2. Remove current yeast plate from refrigerator or cryovial from freezer
3. Start gas burner
4. Flame metal loop until it glows orange
5. Allow loop to cool for 10-15 seconds
6. Scrape a yeast colony from previous yeast plate onto metal loop
7. Gently scrape the inoculating loop across the new agar plate several times
8. Flame loop, rotate plate 60°
9. Scrape the sterile loop through your previous scrape marks (one time only) and continue to spread yeast cells across a clean area of the plate using long strokes with loop
10. Repeat previous step, for a total of 3 series of scrapes across different areas of plate with the last running in the middle of the plate to facilitate single colonies
11. Write the date and your initials on the new plate
12. Place old plate in refrigerator
13. Incubate the new plate at 32°C for 24-48 hours or until colonies are evident
14. Refrigerate new plate with old plate
15. Keep old plate until you make a new plate the next month, so at any one time you have two plates in the refrigerator, one old and one new

2.3 Short-term Yeast Storage Plate Preparation - Streak a new yeast storage plate every 30 days to ensure health of short-term yeast stock

1. Grow yeast from short-term plate in yeast growth medium
2. Grow yeast growth medium to for 48-72 hours
3. Place 0.5 mL yeast growth medium grown to about an abs. of 0.25-0.30 into cryovial with 1 mL glycerol
4. Label cryovial with contents, date, and your initials
5. Place in vial holder
6. Store at -70°C

3.0 PREPARE YES SOLUTIONS

Materials and Equipment:

- Cleaned⁴ spatulas and stir bars
- Cleaned⁴ 1 L, 200 mL volumetric flasks
- Sterile¹ 100 mL glass bottles with screw-top caps⁴
- Sterile¹ 1 L, 250 mL (2) glass bottles with screw-top caps⁴
- Sterile² glass filter supplies (2)
- Sterile¹ 100 mL, 250 mL (2) glass beaker
- Cleaned⁴ 100 mL graduated cylinder

3.1 Minimal Medium Preparation – 90 mL used per assay (~2 plates)

1. Place the following ingredients into a sterile 1-L volumetric flask:

- | | |
|--|--|
| • 1 liter of nanopure H ₂ O | • 30 mg (0.03 g) L-lysine-HCl or |
| • 13.61 g KH ₂ PO ₄ (potassium phosphate monobasic) | 1.642 mL L-lysine-HCl 100 mM soln. |
| • 1.98 g (NH ₄) ₂ SO ₄ (ammonium sulfate) | • 25 mg (0.025 g) L-phenylalanine |
| • 0.2 g MgSO ₄ (magnesium sulfate) or 0.41 g MgSO ₄ * 7 H ₂ O | • 100 mg (0.1 g) L-glutamic acid |
| • 1 mL Fe ₂ (SO ₄) ₃ (ferric sulfate) solution (Prepare in 100 mL volumetric flask) 80 mg Fe ₂ (SO ₄) ₃ & 100 mL nanopure H ₂ O | • 150 mg (0.15 g) L-valine |
| • 50 mg (0.05 g) L-leucine | • 375 mg (0.375 g) L-serine |
| • 50 mg (0.05 g) L-histidine | • 4.2 g KOH (potassium hydroxide) pellets (add last and check pH is ~ 7) |
| • 50 mg (0.05 g) adenine | |
| • 20 mg (0.02 g) L-arginine-HCl | |
| • 20 mg (0.02 g) L-methionine | |
| • 30 mg (0.03 g) L-tyrosine | |
| • 30 mg (0.03 g) L-isoleucine | |

2. Allow solution to mix on stir plate using heat
3. Once all components are fully dissolved, aliquot out 90 mL into glass bottles and put rest in a 1 L glass bottle
4. Place blue plastic lids on bottles, but do not seal lids; leave loose
5. Autoclave bottles with lids at 121°C for 10 minutes
6. Label bottles with chemical name, date, and your initials
7. Store in drawer at room temperature

3.2 Vitamin Solution Preparation – 1 mL of vitamin solution is used per each assay

1. Place the following ingredients into a sterile 250 ml glass beaker:
 - 180 mL nanopure H₂O
 - 8.97 mg (0.00897 g) thiamine
 - 8.0 mg (0.008 g) pyridoxine
 - 8.0 mg (0.008 g) pantothenic acid
 - 40.0 mg (0.04 g) inositol
 - 20 mL biotin solution: (Prepare in 100 mL volumetric flask) 2 mg biotin & 100 mL nanopure H₂O
2. Filter the solution using a 0.2 µm pore size filter into sterile flask then transfer near flame into sterile 250 mL glass bottle and cap
3. Label bottle with chemical name, date, and your initials
4. Store at 4°C

3.3 Glucose Solution Preparation – 20 %w/v – 10 mL of glucose solution used per each assay

1. Place the following ingredients into a sterile 300 ml glass beaker:
 - 44 g C₆H₁₂O₆ * H₂O or 40 g C₆H₁₂O₆ (glucose)
 - 200 mL nanopure H₂O measured using cleaned 100 mL graduated cylinder
2. Filter the solution using a 0.2 µm pore size filter into sterile flask then transfer near flame into sterile 250 mL glass bottle and cap
3. Label bottles with chemical name, date, and your initials
4. Store at room temperature

3.4 L-Aspartic Acid Solution Preparation – 4 mg/mL – 2.5 mL of L-aspartic acid solution is used per each assay

1. Place the following ingredients into a sterile 300 ml glass beaker:
 - 80 mg L-aspartic acid
 - 200 mL of nanopure water measured using cleaned graduated cylinder
2. Pour near flame into sterile 250 mL glass bottle
3. Place blue plastic lids on bottle, but do not seal lids; leave loose
4. Autoclave bottles with lids at 121°C for 10 minutes
5. Label bottle with chemical name, date, and your initials
6. Store at room temperature

3.5 L-Threonine Solution Preparation – 24 mg/mL – 0.8 mL of L-threonine solution is used per each assay

7. Place the following ingredients into a sterile 300 ml glass beaker:
 - 240 mg L-threonine
 - 100 mL of nanopure water measured using cleaned graduated cylinder
8. Pour near flame into sterile 250 mL glass bottle
9. Place blue plastic lids on bottle, but do not seal lids; leave loose
10. Autoclave bottle with lids at 121°C for 10 minutes
11. Label bottle with chemical name, date, and your initials
12. Store at 4°C

3.6 Copper (II) Sulfate Solution Preparation – 20 mM – 250 µL of copper (II) sulfate solution used per each assay

5. Place the following ingredients into a sterile 200 ml glass beaker:
 - 250 mg copper (II) sulfate CuSO_4
 - 50 mL nanopure H_2O measured using cleaned 100 mL graduated cylinder
6. Filter 10 mL aliquots using a 0.2 µm pore size syringe filter near a flame into sterile 100 mL glass bottle and cap
7. Label bottles with chemical name, date, and your initials
8. Store at room temperature wrapped in aluminum foil to prevent light exposure

3.7 Chlorophenol red-β-D-galactopyranoside (CPRG) Solution Preparation - 10 mg/mL - 0.5 mL of CPRG solution is used per each assay

1. Work almost exclusively in a hood with an apron on
2. Place the following ingredients into a sterile 100 mL glass bottle

- 50 mL of nanopure H₂O
 - Container of CPRG
3. Label bottle with chemical name, date, and your initials
 4. Label bottle as "Toxic"
 5. Store at 4°C

3.8 17 β -estradiol stock Solution Standard Preparation - 54,540 ng/L - 100 μ L per row on in the well-plate

1. Place the following directly into a sterile glass storage bottle
 - 0.27724 g 17 β -estradiol
 - 500 mL ethanol
2. This results in a 5.448×10^{-4} g/mL stock 17 β -estradiol solution
3. Mix solution well
4. Place the following ingredients into another sterile glass storage bottle:
 - 1 mL 5.448×10^{-4} g/mL 17 β -estradiol stock solution prepared above
 - 100 mL ethanol
5. Mix solution well
6. This results in a 5.448×10^{-6} g/mL 17 β -estradiol solution.
7. Place the following ingredients into another sterile glass storage bottle:
 - 1 mL 5.448×10^{-6} g/mL 17 β -estradiol 1st dilution solution prepared above
 - 100 mL ethanol
8. This results in the a 5.448×10^{-8} g/mL (54,480 ng/L) final 17 β -estradiol assay solution

4.0 YES ASSAY

Materials and Equipment:

- Sterile² 200 μ L pipette tips (a couple boxes)
- Sterile¹ 250 mL Erlenmeyer flasks
- 30°C incubator
- Solutions prepared in section 3
- 96-well plates

- 96-well plate Reader

4.1 Prepare Yeast Growth Medium

1. Combine the following ingredients in a sterile 250 mL flask:
 - 45 mL minimal medium prepared previously
 - 5 mL 20 % w/v glucose solution
 - 1.25 mL (2*625 μ L) 4mg/mL L-aspartic acid solution
 - 0.5 mL (500 μ L) vitamin solution
 - 0.4 mL (400 μ L) 24 mg/mL L-threonine solution
 - 125 μ L 20 mM copper (II) sulfate solution
2. Remove current yeast growth plate from refrigerator
3. Start gas burner
4. Flame metal loop until it glows orange
5. Allow to cool for 10-15 seconds
6. Scrape a colony from yeast plate onto metal loop
7. Dip metal loop into growth medium in flask
8. Cover flask
9. Incubate at 28-32°C on an orbital shaker until turbid & the absorbance is 0.25-0.30 at 600 nm on plate reader (typical incubation is 36-48 hours)

4.2 Prepare Yeast Assay Medium - If possible prepare yeast assay medium in a type II laminar air flow cabinet.

1. Combine the following ingredients in a sterile 250 mL flask: Each plate requires ~25 mL of assay medium
 - 45 mL minimal medium prepared previously and stored in sterile glass bottles
 - 5 mL 20 % w/v glucose solution
 - 1.25 mL (1250 μ L) 4mg/mL L-aspartic acid solution
 - 0.5 mL (500 μ L) vitamin solution
 - 0.4 mL (400 μ L) 24 mg/mL L-threonine solution
 - 125 μ L 20 mM copper (II) sulfate solution
 - 0.5 mL (500 μ L) Chlorophenol red- β -D-galactopyranoside (CPRG)

- 1.5 ml of the yeast growth medium prepared above that has reached an absorbance of 0.25-0.30 at 600 nm on plate reader. .
- 2. Yeast assay medium is complete; cover with aluminum foil and set aside until needed

4.3 Prepare Dilution Plate - Serial dilutions will be conducted in the dilution microtitre plate. Use one microtitre plate for the serial dilutions and a separate microtitre plate for the actual assay. If possible perform assay in a type II laminar air flow cabinet.

1. Remove two microtitre plates from plastic wrap
2. Label one plate "Assay Plate" and the other "Dilution Plate"
3. Assign rows of dilution plate by writing assigned chemical in black fine-tipped permanent marker on plate cover above the row of interest
4. Dilution plate rows can be assigned as follows:
 - Row A: 17 β -estradiol (E2) standard
 - Row B: E2 standard duplicate
 - Row C: blank
 - Row D-H: assign test chemicals at will
5. Write description of chemicals to be tested and dilution plate row assignments in lab notebook (It's easiest to use abbreviations for writing on plates so it's important to keep track of each solution)
6. Perform a serial dilution as follows (shown for E2 standard for reference)
 1. Place 100 μ L of E2 standard solution into first well (A1) of dilution plate.
 2. Place 50 μ L of methanol (or whatever solvent your test chemicals are dissolved in) into the remaining wells in row A (A2-A12) of the dilution plate
 3. Take 50 μ L from well A1 place into well A2 followed by three pumps of the pipette (in order to mix solutions within the well)
 4. Take 50 μ L from well A2 place into well A3 followed by three pumps of the pipette
 5. Repeat until the first 11 wells in row A contain a different concentration of E2 solution with the last column remaining as a blank

7. Perform serial dilutions using the procedure described above on the remaining solutions in the assigned rows of the dilution plate.

4.4 Prepare Assay Plate

1. Assign rows of assay plate by writing assigned chemical in black fine-tipped permanent marker on plate cover above the row of interest
2. Assay plate rows are assigned exactly as dilution plate rows
3. Using multi-channel pipette place 25 μ L from a row on the dilution plate to the corresponding row on the assay plate
4. Repeat for all 8 rows
5. Place 25 μ L methanol in all blank rows
6. Place cover over dilution plate; it is now waste and can be disposed of accordingly
7. Let contents of all 96 wells in assay plate evaporate by setting assay plate in type II laminar air flow cabinet without cover
8. When wells are dry, add 200 μ L yeast assay medium to all wells
9. Place cover on assay plate
10. Perform Initial Absorbance readings as described in the next section
11. Seal edges of assay plate with autoclave tape
12. Record start time
13. Incubate assay plate in a dark, 32°C naturally ventilated heating cabinet for 3 days

4.5 Plate Absorbance Readings

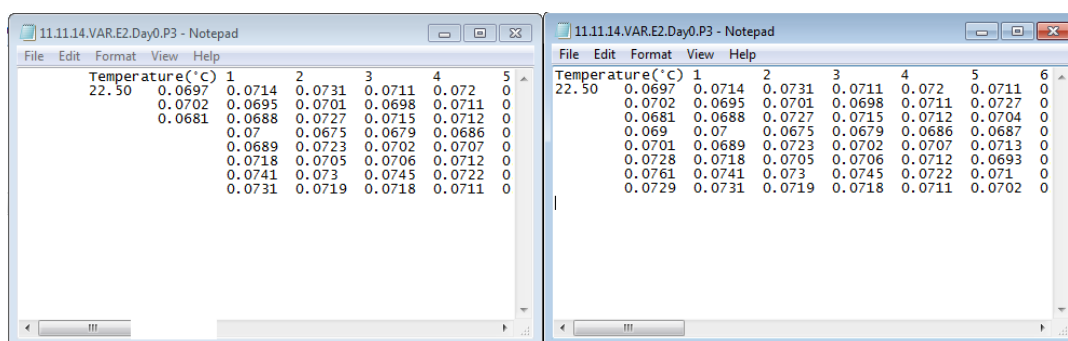
1. Turn on SpectraMax Microplate Reader
2. After incubation period remove plate from incubator
3. Remove autoclave tape from edges of plate
4. Wipe bottom of plate with Kimwipe to remove debris that may interfere with absorbance reading
5. Open SoftMax software and corresponding test file
6. Open plate reader drawer using the software
7. Place plate in plate reader drawer, remove plate cover, and close drawer
8. Have settings adjusted in software file for two absorbance readings (540 nm and 620 nm) as well as 3-5 seconds of shaking time before absorbance reading

9. Press “Read”, plate will be ejected once readings are finished; cover and seal with autoclave tape
10. Save the SoftMax file under the appropriate file for the days readings
11. Turn off plate reader (light bulb will burn out if the plate reader is left on for long periods of time)
12. Allow assay plate to incubate for any additional days, or if finished, dispose of plate accordingly (any solution containing CPRG is considered Hazardous Waste)

5.0 YES ASSAY ANALYSIS

5.1 Transferring SoftMax Readings into Excel

1. Open the SoftMax file on the computer attached to the plate reader.
2. Highlight all of the cells on a given plate and copy (Press CTRL+C) and the paste (CTRL+P) into a text window and save as a .txt file with an appropriate file name including the date, test, and plate number.
3. Do this for each plate within the test being run.
4. Copy .txt files onto a flash drive and transfer to computer with Excel
5. Once the .txt file is opened the blank first tabs that begin each row can be deleted so that text begins each row



6. The whole .txt file can then be highlighted, copied and pasted into an excel spreadsheet

11.11.14.VAR.E2.Day0.P3 - Notepad

Temperature (°C)	1	2	3	4	5	6
22.50	0.0697	0.0714	0.0731	0.0711	0.072	0.0711
	0.0702	0.0695	0.0701	0.0698	0.0711	0.0727
	0.0681	0.0688	0.0727	0.0715	0.0712	0.0704
	0.069	0.07	0.0675	0.0679	0.0686	0.0687
	0.0701	0.0689	0.0723	0.0702	0.0707	0.0713
	0.0728	0.0718	0.0705	0.0706	0.0712	0.0693
	0.0761	0.0741	0.073	0.0745	0.0722	0.071
	0.0729	0.0731	0.0719	0.0718	0.0711	0.0702

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	YES and Extraction Methods Error													
2	Primary Sludge													
3	Plate Started 3/11/14													
4	Day0													
5														
6	Plate 2													
7	Temperat	1	2	3	4	5	6	7	8	9	10	11	12	
8	22.5	0.0697	0.0714	0.0731	0.0711	0.072	0.0711	0.0716	0.0722	0.0713	0.0695	0.0702	0.0711	
9		0.0702	0.0695	0.0701	0.0698	0.0711	0.0727	0.0714	0.0703	0.0705	0.0695	0.0689	0.072	
10		0.0681	0.0688	0.0727	0.0715	0.0712	0.0704	0.0721	0.0708	0.0703	0.0698	0.071	0.0689	
11		0.069	0.07	0.0675	0.0679	0.0686	0.0687	0.0707	0.069	0.0698	0.0678	0.0684	0.0677	
12		0.0701	0.0689	0.0723	0.0702	0.0707	0.0713	0.0706	0.0706	0.0698	0.0713	0.0731	0.0665	
13		0.0728	0.0718	0.0705	0.0706	0.0712	0.0693	0.0694	0.0719	0.0709	0.0686	0.0694	0.0687	
14		0.0761	0.0741	0.073	0.0745	0.0722	0.071	0.0699	0.0716	0.0737	0.0709	0.0709	0.0726	
15		0.0729	0.0731	0.0719	0.0718	0.0711	0.0702	0.0733	0.0696	0.0705	0.0709	0.0718	0.0712	

5.2 Transforming Data for Analysis

1. An excel sheet can be setup as follows:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	YES and Extraction Methods Error																									
2	Primary Sludge																									
3	Plate Started 3/11/14																									
4	Day0																									
5																										
6	Plate 2																									
7	Temperat	1	2	3	4	5	6	7	8	9	10	11	12		1	2	3	4	5	6	7	8	9	10	11	
8	22.5	0.0697	0.0714	0.0731	0.0711	0.072	0.0711	0.0716	0.0722	0.0713	0.0695	0.0702	0.0711		0.0575	0.0607	0.0592	0.0588	0.0617	0.0594	0.0604	0.0613	0.0602	0.058	0.0589	0.0
9		0.0702	0.0695	0.0701	0.0698	0.0711	0.0727	0.0714	0.0703	0.0705	0.0695	0.0689	0.072		0.0581	0.0567	0.0589	0.0571	0.0593	0.0604	0.0579	0.0566	0.0581	0.0581	0.0571	0.0
10		0.0681	0.0688	0.0727	0.0715	0.0712	0.0704	0.0721	0.0708	0.0703	0.0698	0.071	0.0689		0.0574	0.058	0.0597	0.0587	0.0589	0.0599	0.0594	0.0587	0.0582	0.0588	0.0	0.0
11		0.069	0.07	0.0675	0.0679	0.0686	0.0687	0.0707	0.069	0.0698	0.0678	0.0684	0.0677		0.0569	0.0564	0.0574	0.057	0.0565	0.0567	0.0585	0.0588	0.0585	0.057	0.0567	0.0
12		0.0701	0.0689	0.0723	0.0702	0.0707	0.0713	0.0706	0.0706	0.0698	0.0713	0.0731	0.0665		0.0586	0.058	0.0612	0.0593	0.0584	0.0593	0.0591	0.0586	0.0583	0.0589	0.0602	0.0
13		0.0728	0.0718	0.0705	0.0706	0.0712	0.0693	0.0694	0.0719	0.0709	0.0686	0.0694	0.0687		0.0626	0.0603	0.059	0.0591	0.0595	0.0588	0.0577	0.0607	0.0587	0.0571	0.0582	0.0
14		0.0761	0.0741	0.073	0.0745	0.0722	0.071	0.0699	0.0716	0.0737	0.0709	0.0709	0.0726		0.0638	0.0631	0.0621	0.0622	0.0604	0.0612	0.0594	0.0597	0.0628	0.0602	0.0597	0.0
15		0.0729	0.0731	0.0719	0.0718	0.0711	0.0702	0.0733	0.0696	0.0705	0.0709	0.0718	0.0712		0.0611	0.0618	0.0593	0.0595	0.0599	0.0586	0.0606	0.0582	0.0574	0.0604	0.0599	0.0
16													0.06984													0.05
17	Plate 2	Adjusted																								
18	540 nm	1	0.5	0.25	0.125	0.0625	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	620 nm	1	0.5	0.25	0.125	0.0625	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.0
19	E2	-0.00137	0.00156	0.00326	0.00126	0.00216	0.00128	0.00176	0.00238	0.00146	-0.00034	0.00036	0.00128		-0.00061	0.00259	0.00109	0.00069	0.00359	0.00129	0.00229	0.00319	0.00209	-0.00011	0.00079	0.0
20	E2	0.0003625	-0.00034	0.00026	-3.7E-05	0.00126	0.00286	0.00156	0.00046	0.00066	-0.00034	-0.00094	0.00216		-1.3E-05	-0.00141	0.00079	-0.00101	0.00119	0.00229	-0.00021	-0.00151	-1.3E-05	-1.3E-05	-0.00101	0.0
21	Blank	-0.00178	-0.00104	0.00286	0.00166	0.00136	0.00056	0.00226	0.00096	0.00046	-3.7E-05	0.00116	-0.00094		-0.00071	-0.00011	0.0449	0.00189	0.00059	0.00079	0.00179	0.00129	0.00059	8.7E-05	0.00069	-0.0
22	PS-1	-0.00038	0.00016	-0.00234	-0.00194	-0.00124	-0.00114	0.00086	-0.00084	-3.7E-05	-0.00204	-0.00144	-0.00214		-0.00121	-0.00171	-0.00071	-0.00111	-0.00161	-0.00141	0.00039	0.00069	0.00039	-0.00111	-0.00141	-0.0
23	PS-2	0.0002625	-0.00094	0.00246	0.00036	0.00086	0.00146	0.00076	0.00076	-3.7E-05	0.00146	0.00326	-0.00334		0.00049	-0.00011	0.00309	0.00119	0.00029	0.00119	0.00099	0.00049	0.00019	0.00079	0.00209	-0.0
24	PS-3	0.0029625	0.00196	0.00066	0.00076	0.00136	-0.00054	-0.00044	0.00206	0.00106	-0.00124	-0.00044	-0.00114		0.00449	0.00219	0.00089	0.00099	0.00139	0.00069	-0.00041	0.00259	0.00059	-0.00101	8.7E-05	-0.0
25	PS-3	0.0062625	0.00426	0.00316	0.00466	0.00236	0.00116	6.3E-05	0.00176	0.00386	0.00106	0.00106	0.00276		0.00569	0.00499	0.00399	0.00409	0.00229	0.00309	0.00129	0.00159	0.00469	0.00209	0.00159	0.0
26	PS-3	0.0030625	0.00326	0.00206	0.00196	0.00126	0.00036	0.00346	-0.00024	0.00066	0.00106	0.00196	0.00136		0.00299	0.00369	0.00119	0.00139	0.00179	0.00049	0.00249	8.7E-05	-0.00071	0.00229	0.00179	0.0
27																										

2. For initial absorbance reading the adjustment for each individual reading is as follows:

$$Corrected_{initial} = A_{initial} - Average(Column\ 12\ A_0)$$

Where the average of the 12th column absorbance (A) values serves as a blank in order to correct the readings for the whole plate

3. This transformation is for all absorbance readings at 540 and 620 nm initially.
4. For day 3,4, or 5 absorbance reading the transformation equation is different as is as follows:

$$\text{Corrected } A_{620_{final}} = A_{620_{final}} - A_{620_{initial}}$$

$$\text{Corrected } A_{540_{final}} = A_{540_{final}} - A_{540_{initial}} - 1.07 * [\text{Corrected } A_{620_{final}}]$$

This correction is performed for each absorbance reading on the plate.

5. The final corrected 540 nm absorbance values are then going to be used for analysis of estrogenic equivalency

5.3 GraphPad Analysis for Dose-Response

1. Open GraphPad Prism 6.0
2. Under the XY tab click the circle in front of “Dose-response-X is log(dose)” and the Create
3. Under the X column is where all of the logs of the concentrations of samples should be placed
4. Under the GroupA, GroupB etc. columns is each sample grouping. If samples are in triplicate place all three data in one group. If samples are all different place in different groups.
5. Absorbance data can easily be transferred from Excel by copying a row of absorbance data in Excel (CTRL+C) and the pasting the data into GraphPad using (CTRL+T) to transform it from a length of data to a column of data or *vice versa*.
6. Once all data is added into GraphPad click on Analyze under the Analysis toolbar group.
7. Under “XY analyses” click on “Nonlinear regression (curve fit)” and OK
8. Under “Dose-response – Stimulation” click on “log(agonist) vs. response – Variable slope (four parameters)”
9. Under the “Constrain” tab other constraints can be added including conforming the top, bottom and hillslopes of the dose-response curves to a singular shared value so that only the LogEC50 is variable. Or no constraints can be specified.
10. Click OK.
11. A Results and Graphs folder on the left should have pages to click on.
12. EC-50 values from the results page can be used as follows.

$$\text{Sample } EEqs = \left(\frac{E2 \text{ Standard } EC_{50}}{\text{Sample } EC_{50}} \right)$$

Where the E2 Standard units should be in ng/L and the Sample units could be in g/L yielding a Sample EEq (estrogenic equivalent) in units of ng E2/g sample.